SULFATED STEROID GLYCOSIDES FROM THE VIET NAMESE STARFISH *Linckia laevigata*

A. A. Kicha, N. V. Ivanchina, A. I. Kalinovskii, P. S. Dmitrenok, E. V. Sokolova, and I. G. Agafonova

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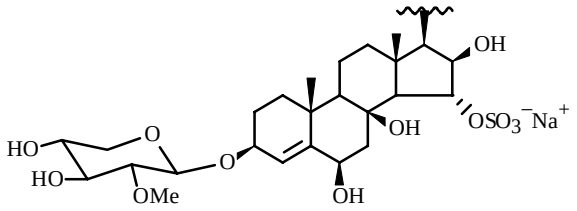
*Five sulfated steriodal compounds including one new glycoside called linckoside L7 (**1**) and four previously known glycosides **2-5** were isolated from the starfish* Linckia laevigata. *The structure sodium (22*E*,24*R*)-3*-O-*(2*-O-*methyl*-β-*D-xylopyranosyl)-29*-O-*(*β-*D-xylopyranosyl)-24-ethylcholest-4,22-dien-3*β*,6*β*,8,15*α*,16*β*,29-hexaol 15*-O-*sulfate was proposed for L7. Linckoside L7 inhibited fertilization and egg-cell development in the sea urchin* Strongylocentrotus intermedius.

Key words: starfish, *Linckia laevigata*, polyhydroxylated steroid, steroid glycoside, sulfate, NMR spectra.

Polyhydroxylated steroids and glycosides related to them are common in starfish as both the free and sulfated forms. These compounds are interesting not only for their unusual chemical structure but also for the variety of biological properties, which include embryotoxic, antifungal, antiviral, antigrowth, and other types of activity [1, 2]. In continuation of the search for new biologically active sulfated steroidal compounds from starfish [3], we studied sulfated steroidal compounds from *Linckia laevigata* (order Valvatida, family Ophiodiasteridae) collected in Van Fong Bay of the South China Sea near the Viet Nam coast.

Column chromatography of the ethanol extract of *L. laevigata* over Polykhrom 1, silica gel, and fluorisil produced fractions of sulfated steroidal compounds from which HPLC over a Diasfer-110-C18 column and rechromatography over a YMC-Pack ODS-A column isolated five pure sulfated steroid glycosides including a new glycoside **1** and four previously known compunds **2-5**.



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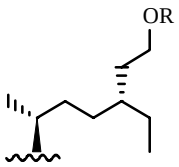

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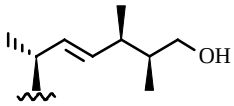

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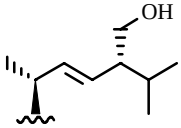

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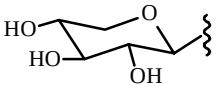

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1: R = A, Δ^{22}; **2:** R = A; **4:** R = H

The known compounds were identified by comparing their PMR and ^{13}C NMR spectra with those published in the literature for echinasteroside F (**2**), which was isolated from the starfish *Echinaster brasiliensis* [4], echinasterosides A (**3**) and B (**4**), and leviusculoside D (**5**), which was obtained previously from the starfish *Henricia leviuscula* [5].

Pacific Institute of Bioorganic Chemistry, Far-East Division, Russian Academy of Sciences, 690022, Vladivostok, pr. 100-Letiya Vladivostoka, 159, fax 7(4232) 31 40 50, e-mail: kicha@piboc.dvo.ru. Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 64-67, January-February, 2007. Original article submitted November 15, 2006.

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TABLE 1. PMR and ^{13}C NMR Spectra of **1** (CD_3OD, δ, ppm, J/Hz)[a]

Atom	δ_C[b]	δ_H[c]	HMBC
1	39.7 CH_2	1.78 (m)	
		1.27 (m)	
2	27.9 CH_2	1.96 (m)	
		1.76 (m)	
3	77.5 CH	4.18 (m)	
4	126.8 CH	5.63 (br.s)	C-6
5	148.5 C		
6	76.3 CH	4.29 (t, 2.9)	C-8, C-10
7	44.0 CH_2	2.65 (dd, 3.1, 15.0)	C-6, C-8, C-9
		1.46 (dd, 3.0, 14.5)	
8	76.0 C		
9	57.6 CH	1.03 (m)	
10	37.7 C		
11	19.5 CH_2	1.90 (m)	
		1.49 (m)	
12	42.7 CH_2	1.97 (m)	
		1.22 (m)	
13	44.7 C		
14	62.0 CH	1.22 (d, 10.6)	C-15
15	87.4 CH	4.77 (dd, 2.0, 10.7)	C-8, C-14, C-16
16	80.4 CH	4.27 (dd, 2.2, 7.8)	C-13
17	60.9 CH	1.30 (m)	
18	17.0 CH_3	1.22 (s)	C-12, C-13, C-14, C-17
19	22.6 CH_3	1.36 (s)	C-1, C-5, C-9, C-10
20	34.4 CH	2.56 (m)	
21	20.6 CH_3	1.04 (d, 7.0)	C-17, C-20, C-22
22	139.9 CH	5.49 (dd, 7.7, 15.3)	C-20, C-24
23	130.3 CH	5.15 (dd, 9.3, 15.3)	C-20
24	46.9 CH	1.96 (m)	
25	33.5 CH	1.50 (m)	
26	19.5 CH_3	0.83 (d, 7.0)	C-24, C-25, C-27
27	21.3 CH_3	0.88 (d, 7.0)	C-24, C-25, C-26
28	33.9 CH_2	1.77 (m)	
		1.42 (m)	
29	69.6 CH_2	3.82 (m)	
		3.47 (m)	
1′	104.6 CH	4.41 (d, 7.6)	C-3, C-3′
2′	84.9 CH	2.81 (dd, 7.6, 9.1)	C-1′, C-3′, OMe
3′	77.5 CH	3.31[d] (t, 8.6)	
4′	71.2 CH	3.46 (m)	C-5′
5′	66.8 CH_2	3.80 (dd, 5.4, 11.5)	C-3′, C-4′
		3.16 (t, 11.5)	C-1′, C-3′, C-4′
2′-OMe	61.2 CH_3	3.57 (s)	C-2′
1″	105.2 CH	4.25 (d, 7.7)	C-29
2″	75.0 CH	3.12 (dd, 7.7, 8.9)	C-1″, C-3″
3″	77.8 CH	3.38 (t, 8.8)	C-2″
4″	71.5 CH	3.43 (m)	C-5″
5″	66.8 CH_2	3.81 (dd, 5.0, 11.5)	C-1″
		3.33[d] (t, 11.3)	

[a]PMR and ^{13}C NMR spectra recorded at 500 and 125.8 MHz, respectively; [b]multiplicity of signals determined using DEPT spectra; [c]signals assigned using 2D ^1H-^1H COSY 45 and HSQC NMR spectroscopy; [d]SSCC determined using 1D TOCSY spectrum.

The high-resolution mass spectrum (ESI-TOF) of **1** (linckoside L7) contained a peak for a pseudomolecular ion with *m/z* 895.3768 $[M_{Na} + Na]^+$, which corresponded to the molecular formula $C_{40}H_{65}O_{17}SNa$. PMR and ^{13}C NMR spectra of **1** (Table 1) and echinasteroside F (**2**) [4] indicated that **1**, like **2**, contained the identical Δ^4-3β,6β,8,15α,16β,29-hexahydroxysteroidal aglycon with a C-15 sulfoxy group, a C-3 2-*O*-methyl-β-D-xylopyranose, and a C-29 β-D-xylopyranose. However, in contrast with **2**, the NMR of **1** contained a signal for an additional double bond in the aglycon sidechain.

According to ^{13}C NMR and DEPT spectra, the sidechain of **1** contained three methyls, two methylenes including one C atom bound to O (δ 69.9 ppm), three methines, and two C atoms of a double bond. All H and C signals of the steroid nucleus and sidechain of **1** were determined using ^1H—^1H COSY 45, HSQC, and HMBC experiments (Table 1). The SSCC $J_{22,23}$ = 15.3 Hz was consistent with the *trans*-configuration for a 22(23)-double bond [6]. The spectral results suggested that **1** contained the rather rare 29-*O*-(β-D-xylopyranosyl)-22-en-24-ethylcholestane sidechain, which has been encountered earlier only in attenuatoside S-II from the starfish *Hacelia attenuata* [6].

Mild sulfolytic decomposition of **1** gave the desulfated derivative **1a**. The signal for H-15 in its PMR spectrum (see Experimental) was shifted to strong field from δ 4.77 to 4.16 ppm compared with the spectrum of **1**. This also indicated that the sulfoxy group was on C-15. HMBC correlations of the anomeric protons and H-1′/C-3 and H-1″/C29 atoms confirmed that **1** had a C-3 2-*O*-methyl-β-xylopyranose and a C-29 β-xylopyranose.

The configuration of asymmetric C-24 was proposed as *R* in analogy with echinasteroside F. Based on existing data, linckoside L7 was assigned the structure sodium (24*R*)-3-*O*-(2-*O*-methyl-β-D-xylopyranosyl)-29-*O*-(β-D-xylopyranosyl)-24-ethyl-5α-cholest-4,22-dien-3β,6β,8,15α,16β,29-hexaol 15-*O*-sulfate. Thus, it was found that **1** is the Δ^{22}-analog of echinasteroside F [4].

Unsulfated monosides and biosides containing mainly the Δ^4-3β,6β,8,15α,16β-pentahydroxysteroid nucleus with 2-*O*-methyl-β-D-xylopyranose or β-D-xylopyranose on C-3 were isolated earlier in a study of the *L. laevigata* population near Okinawa (Japan) [7]. Judging from our results, the streoid fraction from the Viet Namese harvest of *L. laevigata* had structurally similar but more polar **1-5** with the 15α-sulfate.

The cytotoxic activity of **1** was investigated in two cell models. The first used fertilized egg cells of the sea urchin *Strongyocentrotus intermedius* at the eight blastomer stage. Linckoside L7 exhibited weak cytotoxic activity, reaching 100% inhibition of egg-cell division (ED_{100}) at 50 μg/mL. The second model was a sperm-test in which L7 demonstrated moderate cytotoxic activity, reaching 100% inhibition of egg-cell fertilization for *S. intermedius* (ED_{100}) after preliminary treatment of spermatosoids with the glycoside at 25 μg/mL.

EXPERIMENTAL

PMR and ^{13}C NMR spectra were recorded on Bruker DPX 300 (300 and 75.5 MHz, respectively) and DRX 500 (500 and 125.8 MHz, respectively) spectrometers with TMS internal standard. Optical rotations were measured on a Perkin—Elmer 343 polarimeter.

High-resolution ESI-TOF mass spectra were measured on a Micromass Q-TOF Micro spectrometer (Micromass, England) using electrospray ionization. The instrument was calibrated for high-resolution measurements using a mixture of polyethyleneglycols with molecular weights from 200 to 1000 (resolving power 5000, deviation <5 ppm).

MALDI-TOF mass spectra were measured on a Biflex III mass spectrometer (Bruker, Germany, N_2-laser, 337 nm). Samples were dissolved in MeOH (1 mg/mL). Aliquots (1 μL) were analyzed in a matrix of 2,5-dihydroxybenzoic acid. HPLC was carried out in an Agilent 1100 Series chromatograph (refractometry detector).

Column chromatography used Polykhrom 1 (Teflon powder, Biolar, Latvia), silica gel KSK (50-160 μm, Sorbpolimer, Krasnodar, Russia), and fluorisil (200-300 mesh, Aldrich Chemical Co.). TLC was carried out on Sorbfil plates (4.5 × 6.0 cm, Sorbpolimer, Krasnodar, Russia) with a layer of silica gel CTX-1A (5-17 μm) fixed on the foil. Compounds were developed by spraying with conc. H_2SO_4 and subsequent heating at 110°C for 10 min.

Starfish were collected in January 2005 in the South China Sea (Van Fong Bay, Viet Namese coast) at 5-10 m depth during the 30th scientific expedition of the Research Vessel Akademik Oparin. Starfish were determined visually by V. B. Krasokhin (Pacific Inst. Bioorg. Chem., FED, RAS, Valdivostok, Russia).

Isolation of 1-5. Ground starfish (3.6 kg) were extracted twice with ethanol (3 L/kg) at room temperature. The ethanol extracts were concentrated in vacuo. The residue was dissolved in water (1.5 L) and passed through a column (8 × 62 cm) of

Polikhrom 1. The column was rinsed with water until the effluent did not contain chloride ions and then with ethanol (50%). The ethanol effluent was evaporated. The resulting total fraction of steroidal compounds (5.5 g) was chromatographed over a column of silica gel (4 × 18 cm) using $CHCl_3$:C_2H_5OH (stepwise gradient, 4:1→1:6) to afford a fraction containing sulfated steroidal compounds according to TLC (504 mg, R_f 0.48-0.71, butanol:ethanol:water, 4:1:2). Then the fraction was separated over a column of fluorisil (2.5 × 15 cm) using $CHCl_3$:C_2H_5OH (stepwise gradient, 4:1→1:2). The resulting subfractions were purified by HPLC over Diasfer-110-C18 (10 μm, 15 × 250 mm) with elution by ethanol (65%) and rechromatography over a column of YMC-Pack ODS-A (5 μm, 10 × 250 mm) with elution by methanol (75%) to afford **1** (2.5 mg, R_f 0.48), **2** (15 mg, R_f 0.62), **3** (3.8 mg, R_f 0.71), **4** (6.9 mg, R_f 0.71), and **5** (2.6 mg, R_f 0.68).

Linckoside L7 (1): amorphous compound, $[\alpha]_D^{20}$ -20.4° (*c* 0.25, MeOH). Table 1 gives the PMR and ^{13}C NMR spectra. HR ESI-TOF(+) mass spectrum (*m*/*z*): 895.3768 $[M_{Na} + Na]^+$ (calc. for $C_{40}H_{65}O_{17}SNa_2$, 895.3738).

Echinasteroside F (2): amorphous compound, $[\alpha]_D^{20}$ -13.5° (*c* 0.30, MeOH). MALDI/TOF(+) mass spectrum (*m*/*z*): 897 $[M_{Na} + Na]^+$; MALDI/TOF(-) mass spectrum (*m*/*z*): 851 [M - cation]$^-$. PMR and ^{13}C NMR spectra were identical to those reported [4].

Echinasteroside A (3): amorphous compound, $[\alpha]_D^{20}$ -14.4° (*c* 0.30, MeOH). MALDI/TOF(+) mass spectrum (*m*/*z*): 749 $[M_{Na} + Na]^+$. PMR and ^{13}C NMR spectra were identical to those reported [5].

Echinasteroside B (4): amorphous compound, $[\alpha]_D^{20}$ -10.2° (*c* 0.30, MeOH). MALDI/TOF(+) mass spectrum (*m*/*z*): 765 $[M_{Na} + Na]^+$. PMR and ^{13}C NMR spectra were identical to those reported [5].

Leviusculoside D (5): amorphous compound, $[\alpha]_D^{20}$ -16.2° (*c* 0.17, MeOH). MALDI/TOF(+) mass spectrum (*m*/*z*) 749 $[M_{Na} + Na]^+$; MALDI/TOF(-) mass spectrum (*m*/*z*): 703 [M - cation]$^-$. The PMR spectrum was identical to that reported [5]. ^{13}C NMR spectrum (125.8 MHz, CD_3OD, δ, ppm): (aglycon) 39.6 (C-1), 27.9 (C-2), 77.5 (C-3), 126.8 (C-4), 148.5 (C-5), 76.3 (C-6), 44.0 (C-7), 76.0 (C-8), 57.6 (C-9), 37.7 (C-10), 19.5 (C-11), 42.8 (C-12), 44.7 (C-13), 62.0 (C-14), 87.3 (C-15), 80.2 (C-16), 61.2 (C-17), 17.0 (C-18), 22.6 (C-19), 34.4 (C-20), 20.2 (C-21), 140.6 (C-22), 128.4 (C-23), 53.3 (C-24), 29.4 (C-25), 10.2 (C-26), 21.5 (C-27), (carbohydrate) 104.6 (C-1′), 84.9 (C-2′), 77.5 (C-3′), 71.2 (C-4′), 66.8 (C-5′), 61.2 (2′-OMe).

Desulfation of 1. Compound **1** (1.5 mg) was heated with dioxane:pyridine (1:1, 1 mL) at 100°C for 4 h. The mixture was evaporated in vacuo. The solid was chromatographed over a column of fluorisil (1 × 3 cm) using $CHCl_3$:C_2H_5OH (3:2) to afford **1a** (0.8 mg). MALDI/TOF(+) mass spectrum (*m*/*z*): 793 $[M + Na]^+$. PMR spectrum (500 MHz, CD_3OD, δ, ppm, J/Hz): (algycon) 0.84 (3H, d, J = 7.0, CH_3-26), 0.88 (3H, d, J = 7.0, CH_3-27), 1.04 (1H, d, J = 7.0, CH_3-21), 1.16 (3H, s, CH_3-18), 1.36 (3H, s, CH_3-19), 1.50 (1H, dd, J = 3.0, 14.8, H-7a), 2.58 (1H, dd, J = 3.0, 15.0, H-7e), 3.47 (1H, m, H′-29), 3.82 (1H, m, H-29), 3.92 (1H, dd, J = 2.5, 7.3, H-16), 4.16 (1H, dd, J = 2.5, 10.8, H-15), 4.18 (1H, m, H-3), 4.31 (1H, t, J = 3.0, H-6), 5.24 (1H, dd, J = 9.2, 15.3, H-23), 5.49 (1H, dd, J = 7.8, 15.3, H-22),5.64 (1H, br.s, H-4), (carbohydrate) 4.41 (1H, d, J = 7.5, H-1′), 2.81 (1H, dd, J = 7.6, 9.1, H-2′), 3.25-3.36 (H-3′, under solvent signal), 3.45 (1H, m, H-4′), 3.17 (1H, t, J = 11.3, H-5′), 3.80 (1H, dd, J = 5.4, 11.5, H-5′), 3.57 (3H, s, OMe), 4.16 (1H, d, J = 7.6, H-1″), 3.14 (1H, m, H-2′), 3.25-3.36 (H-3″, H-5″, under solvent signal), 3.45 (1H, m, H-4′), 3.84 (1H, dd, J = 5.3, 11.3, H-5″).

Biological Test. The cytotoxic activity of **1** was tested using the ability to inhibit fertilization of egg cells and development of embryos of *S. intermedius* according to the literature methods [8].

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